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Acute rejection in heart transplant patients is associated with the presence of committed donor-specific cytotoxic lymphocytes in the graft but not in the blood

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SUMMARY

In vivo-activated, committed donor-specific cytotoxic lymphocytes (cCTL) can be propagated and expanded from endomyocardial biopsies (EMB) in IL-2-enriched medium especially during an acute rejection episode. We report here our efforts to detect these cCTL by the same technique in peripheral blood at the moment of rejection and when no rejection was diagnosed. During or just before rejection, significantly less frequent ($P < 0{\cdot}01$) donor reactive cCTL were found in PBL samples (two out of 20) than in the simultaneously taken EMB samples (13 out of 19). Donor B-LCL and/or third-party B-LCL were lysed by 15 PBL samples. Inhibition studies revealed that this lysis was due to LAK-like cytotoxicity. The results show that peripheral blood does not reflect intra-graft events, which is probably the reason for the irreproducible results of diagnosis of rejection by monitoring immunological parameters in the peripheral blood.

Keywords immunological monitoring cCTL graft-infiltrating cells heart transplantation peripheral blood lymphocytes

INTRODUCTION

In the last decade several attempts have been made to correlate immunological parameters of peripheral blood lymphocytes (PBL) with acute cellular rejection after solid organ transplantation. Recently, investigators have tried to demonstrate the presence of activated T cells in the PBL or increased levels of soluble IL-2 receptor (sIL-2R) as a marker of T cell activity during acute rejection. Activated T cells can be distinguished from resting T cells by their morphological appearance and by their expression of IL-2 receptors and HLA-DR molecules. Several investigators reported that neither flow cytometry, nor cyto-immunological monitoring based on morphology (CIM) or serial sIL-2R monitoring were suitable tools to diagnose intra-graft events reliably in the peripheral blood [1–6]. In other studies, however, activated T cells were found in PBL during graft rejection [7–10]. We wondered whether these activated T cells are *in vivo*-activated, committed, donor-directed cytotoxic T cells (cCTL) that are responsible for rejection. Such cCTL can be propagated from kidney biopsies and from endomyocardial biopsies (EMB) after transplantation, and their presence seems to correlate with histological rejection [11–13].

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Here we describe our efforts to culture these donor-reactive cCTL from PBL. The functional capacity of these cells was compared with the reactivity of biopsy-derived graft-infiltrating lymphocytes.

MATERIALS AND METHODS

In our centre a total of 535 EMB from 87 patients obtained at several time intervals after heart transplantation were cultured in IL-2-containing medium [13]. Peripheral blood (40 ml) was taken from most patients simultaneously with each biopsy. For the current study we selected 39 PBL and 38 EMB samples from 20 cardiac transplant recipients. EMB and PBL from 13 patients were studied in the first half year after heart transplantation. Six patients were studied during and around a biopsy-proved rejection episode, and seven at a period during which no rejection was monitored. From nine of these patients, two to seven consecutive time-points and from four patients one time-point were studied. EMB and PBL from seven patients taken more than 1 year after heart transplantation were studied. All patients had received preoperative blood transfusions and were under immunosuppression with cyclosporine A and low-dose steroids. Rejection was monitored by histological examination of EMB, and the histological rejection grade was assessed

according to Billingham's criteria [14]: grade 0, no evidence of rejection; grade 1, mild rejection, perivascular and endocardial infiltration with pyroninophylic lymphocytes, endocardial and interstitial oedema; and grade 2, moderate rejection, more dense perivascular interstitial infiltrates, and focal myocytolysis (necrosis). Grade 3, severe rejection, was never observed in our study. Rejection therapy, methylprednisolone, OKT-3 or rabbit anti-thymocyte-globulin was only instituted in case of moderate or severe rejection.

T cell lines from EMB

T cell lines were generated from EMB as described [13]. Briefly, biopsy fragments were placed in 200 μl of culture medium (CM) containing 10% (v/v) ($\pm$60 U IL-2) lectin-free Lymphocult (Biotest, Dreiech, Germany), (CM-IL-2), in the presence of 10^5 irradiated (40 Gy) autologous PBL. CM consisted of RPMI 1640 Dutch modification (GIBCO, Paisley, UK) supplemented with 10% human serum, L-glutamine, penicillin and streptomycin. Biopsy cultures were grown at 37°C in a humidified CO_2 incubator. Cultures were assayed for phenotypic expression of differentiation antigens and donor specific cytotoxicity within 4 weeks after initiation of the culture.

T cell lines from PBL

Buffy coats were harvested from blood after centrifugation at 600 ***g*** for 7 min and diluted with an equal volume of HBSS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. This suspension was layered on a Ficoll–Isopaque gradient ($\delta = 1{\cdot}077$) (Pharmacia, Uppsala, Sweden) and centrifuged for 20 min at 800 ***g*** at room temperature. Lymphocytes were harvested from the interface, washed three times and cultured in 200 μl CM-IL-2 in 96-well round-bottomed tissue microtitre plates (Costar 3799, Cambridge, MA). From each sample 10–20 wells containing 10^5 cells were grown for 10–14 days at 37°C in a humidified 5% CO_2 incubator. Half of the culture medium was changed every 2–3 days.

Target cells

To determine allospecific cytotoxicity, PHA-transformed T blasts (T-BL) and EBV-transformed B lymphoblast cell lines (B-LCL) from donor origin were used. T-BL were used as targets for HLA class I-specific cytotoxicity. Since HLA class II antigen expression on T-BL is usually low, B-LCL were used as targets for HLA class II-specific reactivity. Third-party B-LCL or T-BL (sharing no HLA antigens with the donor) served as negative controls. As control for non-MHC-restricted (NK and/or LAK) cytotoxicity, the proerythro-blastic tumour cell line K562 was used.

For the generation of T-BL, 10^7 nucleated donor spleen cells/ml were stimulated for 3 days with 1% PHA-M (Difco, Detroit, MI) in CM and then further cultured for 3 days in CM supplemented with 5% Lymphocult (Biotest) as a source of IL-2. Generation of B-LCL was done as described elsewhere [15]. Briefly, lymphocytes isolated from donor spleen or PBL were resuspended in culture supernatant of the marmoset cell line B95-8 and incubated for 4 h at 37°C in 5% CO_2. After washing, cells were resuspended in CM containing 1% PHA and 5% heat-inactivated fetal calf serum (FCS) and cultured.

Cytotoxicity assay

Cytotoxic capacity of the cultures was measured as described before [13]. Briefly, $2{\cdot}5 \times 10^3$ ^{51}Cr-labelled PHA blasts or B-LCL were mixed with effector cells in effector-to-target (E:T) ratios ranging from 40:1 to 1:1 in 200 μl/well CM in 96-well U-bottomed microtitre plates (Costar). The plates were centrifuged (60 ***g***, 1 min) and incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2. Supernatants were collected with a Skatron harvesting system (Skatron, Lier, Norway). The percentage of specific lysis was calculated according to the formula:

$$\frac{\text{Experimental} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100$$

Maximal release was determined from a Triton $\times$ 100 lysate of the target cells. Spontaneous release was determined by incubation of target cells in CM only.

A CML was considered positive when the percentage specific lysis exceeded 10% and the slope of the ratio curve was positive.

Cold target inhibition

To discriminate between *in vivo*-induced MHC-restricted donor-specific reactivity and culture-induced non-specific cytotoxicity (LAK), cold target inhibition studies were performed. Unlabelled (cold) K562 cells were mixed in 10-fold excess with ^{51}Cr-labelled B-LCL target cells or ^{51}Cr-labelled K562. Control values were established by adding donor or third-party T-BL as cold competitors.

RESULTS

After culture in CM with 60 U IL-2 for 10–14 days, PBL samples were harvested and analysed for cytotoxic function. Cultures propagated from EMB in the same medium were analysed after 3–4 weeks of culture.

Cytotoxic activity

PBL and EMB cultures were tested in the CML assay with donor T-BL, donor B-LCL, third-party B-LCL and T-BL, mismatched with the donor, and K562 as targets. As shown in Tables 1–3, only two cultured PBL samples did lyse donor T-BL at a low but significant level. Third-party T-BL were never killed (data not shown). The results obtained with B-LCL as targets had a more variable pattern. Twelve cultures lysed both donor and third-party B-LCL, seven killed either donor B-LCL or third-party B-LCL and 15 cultures did not lyse either. Five samples were not tested on all targets. Since the PBL samples that killed B-LCL always had high cytotoxicity against the LAK-sensitive cell line K562 (Fig. 1) B-LCL killing could be due to LAK activity induced by IL-2 during culture. As shown by Oshimi *et al.* [16], B cell blasts are more sensitive to IL-2-activated LAK effectors than T cell blasts. Only one of the corresponding T cell cultures propagated from the EMB exhibited LAK-like cytotoxicity, a third-party B-LCL and K562 were lysed at the same level (Table 1). All other EMB-derived T cell lines tested lysed neither third party B-LCL (Tables 1 and 2) nor K562 (data not shown).

To distinguish between aspecific LAK activity and donor-specific class II alloreactivity, cold target inhibition was performed with a 10-fold excess of unlabelled K562 added to ^{51}Cr-labelled donor B-LCL, third-party B-LCL or K562. In culture

Table 1. Cytotoxic activity of EMB- and PBL-derived cell lines obtained in the first half year after heart transplantation during the period in which patients had an acute rejection episode

		EMB		PBL		
		CML‡				
Sample identification*	Rejection grade†	DO	3P	DO-T-BL§	DO-BLCL¶	3P-BLCL‖
KU22	1	83	38	0	19 (0)	15 (0)
KU29	0	71	4	0	25 (0)	— (—)
KU42	2	78	6	20	32 (20)	0 (—)
KU52	1	74	5	0	25 (0)	30 (5)
KU57	1	NG	—	20	— (—)	— (—)
KU66	1	NG	—	0	0 (—)	90 (—)
KU94	2	48	8	0	31 (4)	30 (—)
RO7	0	NG	—	0	15 (5)	20 (0)
RO14	2	46	3	0	0 (—)	0 (—)
RO28	1	46	2	0	0 (0)	0 (0)
RO33	2	74	3	0	0 (0)	0 (0)
RO40	1	75	9	0	0 (0)	0 (0)
RO47	1	75	7	0	0 (0)	4 (10)
OO102	2	—	—	0	10 (0)	0 (0)
OO149	1	NG	—	0	45 (6)	42 (5)
EN41	1	NG	—	0	0 (—)	0 (—)
EN51	2	64	1	0	0 (—)	0 (—)
WO13	1	NG	—	0	0 (—)	0 (—)
WO18	2	36	5	—	— (—)	— (—)
KO57	2	40	6	0	— (—)	— (—)

*Patient identification, followed by the number of days after heart transplantation.

†Billingham's rejection grade [14].

‡Percentage donor-specific lysis (Do) and lyses of third-party cells (3P), at an E:T ratio of 20.

§Percentage lysis of donor PHA-blasts at an E:T ratio of 20.

¶Percentage lysis of donor B-LCL at an E:T ratio of 20 before and, in parentheses, after inhibition with 10-fold excess cold K562 targets at an E:T ratio of 5.

‖Percentage lysis of unrelated third-party B-LCL before and, in parentheses, after inhibition with 10-fold excess of cold K562.

—, No sample or not tested; NG, no or insufficient growth.

KU42 alone the reactivity against donor B-LCL could not be inhibited by cold K562 (Fig. 2a). This was one of the two cultures that specifically lysed donor T-BL (Table 1). The lysis of ^{51}Cr-labelled K562 by this culture was blocked by cold K562 up to a E:T ratio of 10, whereas a 10-fold excess of unlabelled third-party T-BL had no influence (Fig. 2b). In all other PBL cultures reactivity against B-LCL could be inhibited by cold K562 and not by cold third-party T-BL. In Fig. 3 a representative example of these experiments is depicted. Inhibition was not always complete over the whole E:T ratio range, because of high LAK activity. Cultures were considered not to be donor specific when the percentage lysis at an E:T ratio of 5 was reduced by cold K562 to 10% or less. The PBL cultures BN25, PA741, PJ844, and TW592 contained too few effector cells to perform these control tests. Since reactivity against donor T-BL was negative and the reactivity against third-party B-LCL and K562 was high, we regarded the lysis of B-LCL by the PBL cultures BN25 (Table 2) and TW592 (Table 3) as caused by aspecific LAK activity.

Relation between cCTL and rejection

In order to clarify whether donor-reactive cCTL were detectable in PBL during rejection, we expanded PBL *in vitro* from a group of six patients during an episode in which they showed a biopsy proved rejection crisis. During these episodes cCTL are generally found in the graft (Table 1). Donor-reactive CTL were found significantly less often in PBL samples (two out of 20) than in EMB samples simultaneously taken (13 out of 19) (χ^2-test, $P<0{\cdot}01$).

It can be argued that at the time of a rejection crisis cCTL accumulate in the graft, which results in an extremely low cCTL frequency in the PBL. If cCTL circulate, it might well be that the frequency of donor-specific cCTL in PBL will be higher during periods in which less cCTL are present in the graft. We therefore

Table 2. Cytotoxic activity of EMB- and PBL-derived cell lines obtained in the first half year after heart transplantation during a period in which patients had no acute rejection episode

Sample identification*	EMB			PBL		
	Rejection grade†	CML‡				
		DO	3P	DO-T-BL§	DO-BLCL¶	3P-BLCL‖
CL159	—	—	—	0	25 (0)	54 (15)
CL177	1	NG	—	0	35 (9)	15 (0)
CL219	0	NG	—	0	52 (2)	— (—)
LA117	0	0	0	—	0 (—)	30 (—)
LA169	—	—	—	0	0 (—)	70 (—)
LA186	0	61	6	0	30 (—)	40 (—)
WL97	1	44	4	0	0 (—)	0 (—)
WL132	0	NG	—	0	48 (—)	65 (—)
JU26	1	42	7	0	0 (—)	0 (—)
JU32	0	NG	—	0	0 (—)	0 (—)
BN25	1	88	0	0	30 (—)	60 (—)
JO29	0	NG	—	0	22 (0)	0 (0)
BU149	1	56	2	0	0 (—)	0 (—)

*Patient identification, followed by the number of days after heart transplantation.

†Billingham's rejection grade [14].

‡Percentage donor specific lysis (Do) and lyses of third party cells (3P), at an E:T ratio of 20.

§Percentage lysis of donor PHA-blasts at an E:T ratio of 20.

¶Percentage lysis of donor B-LCL at an E:T ratio of 20 before and, in parentheses, after inhibition with 10-fold excess cold K562 targets at an E:T ratio of 5.

‖Percentage lysis of unrelated third-party B-LCL before and, in parentheses, after inhibition with 10-fold excess of cold K562.

—, No sample or not tested; NG, no or insufficient growth.

Table 3. Cytotoxic activity against donor cells of EMB and PBL-derived cell lines obtained more than 1 year after heart transplantation, during a period in which patients had no acute rejection episode

Sample identification*	EMB		PBL		
	Rejection grade†	CML‡	DO-T-BL§	DO-BLCL¶	3P-BLCL‖
PA741	0	NG	0	— (—)	— (—)
PJ844	0	NG	0	— (—)	0 (—)
LI844	1	NG	0	0 (—)	0 (—)
ST662	0	NG	0	0 (—)	— (—)
KR1290	0	NG	0	0 (—)	0 (—)
TW592	1	NG	0	71 (—)	80 (—)
BL1582	0	NG	0	20 (0)	70 (18)

*Patient identification, followed by the number of days after heart transplantation.

†Billingham's rejection grade [14].

‡Percentage donor-specific lysis at an E:T ratio of 20.

§Percentage lysis of donor PHA blasts at an E:T ratio of 20.

¶Percentage lysis of donor B-LCL at an E:T ratio of 20 before and, in parentheses, after inhibition with 10-fold excess cold K562 targets at an E:T of 5.

‖Percentage lysis of unrelated third-party B-LCL before and, in parentheses, after inhibition with 10-fold excess of cold K562.

—, No sample or not tested; NG, no or insufficient growth.

analysed PBL samples from a period during which no rejection crisis occurred. Table 2 shows the results obtained with PBL and EMB taken from seven other patients in a comparable period after heart transplantation to the first group, but this time during a period of freedom from rejection. However, none of the PBL cultures lysed donor T-BL. Activity against donor B-LCL could always be inhibited by cold K562 and not by cold third-party T-BL (Fig. 3a).

Table 3 summarizes results obtained from cultured EMB and PBL taken from seven additional patients, more than 1 year after heart transplantation. Also in this period, when most patients do not encounter a rejection episode any more and EMB did not contain donor reactive cCTLs, no cCTL could be propagated from the blood.

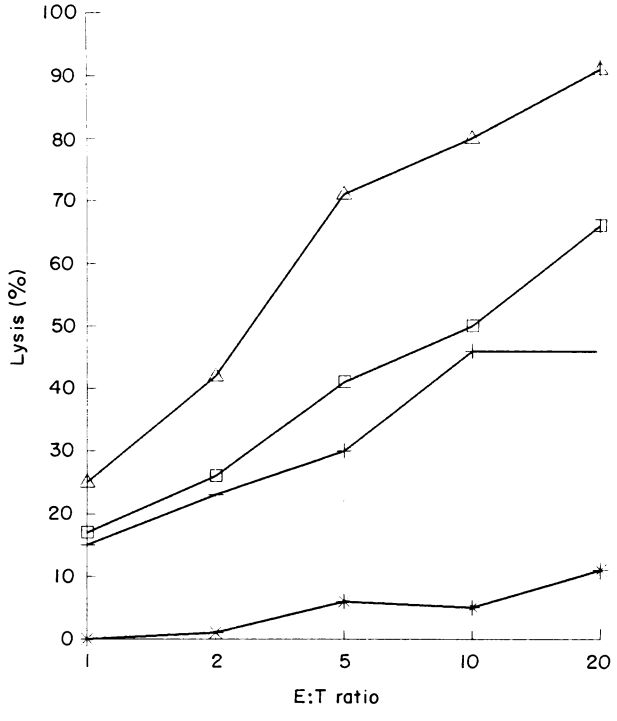

Fig. 1. A representative experiment showing the LAK-like cytotoxicity pattern of PBL culture WL132 grown for 12 days on IL-2, tested in a 4-h ^{51}Cr release assay against donor T-BL (*) and B-LCL(+), third-party B-LCL (□) and K562 (△).

DISCUSSION

Many attempts have been made to diagnose rejection using peripheral blood parameters and thus replace the need to biopsy the graft. Parameters such as changes of T cell subsets or their ratio, an increase in $CD25^+$ or $HLA\text{-}DR^+$ T cells, sIL-2R levels or morphological changes in PBL have been studied [1–6, 17–20]. Results have been variable and inconclusive.

The aim of our study was to verify whether donor-specific immunological reactivity of primed cells in PBL reflects intra-graft events. For that purpose we compared the occurrence of donor-reactive cCTL in the peripheral blood and in the graft, in episodes with rejection and during immunological quiescence. Activated, antigen-specific, committed CTL are derived from precursor CTL (pCTL). The activation process resulting in proliferation and maturation of the CTL requires two signals, alloantigen and cytokines [21]. If pCTL have recently received the alloantigenic signal *in vivo*, only IL-2 is required for subsequent *in vitro* growth during 3–4 weeks without loss of specificity [11–13]. Thus if recently alloactivated cCTL are present in the PBL it should be possible to expand them in the presence of IL-2 without loss of specific function. Our results, however, demonstrated that such cells were not detectable within PBL population of most patients studied, neither at the time of a biopsy-proved rejection when cCTL were present in

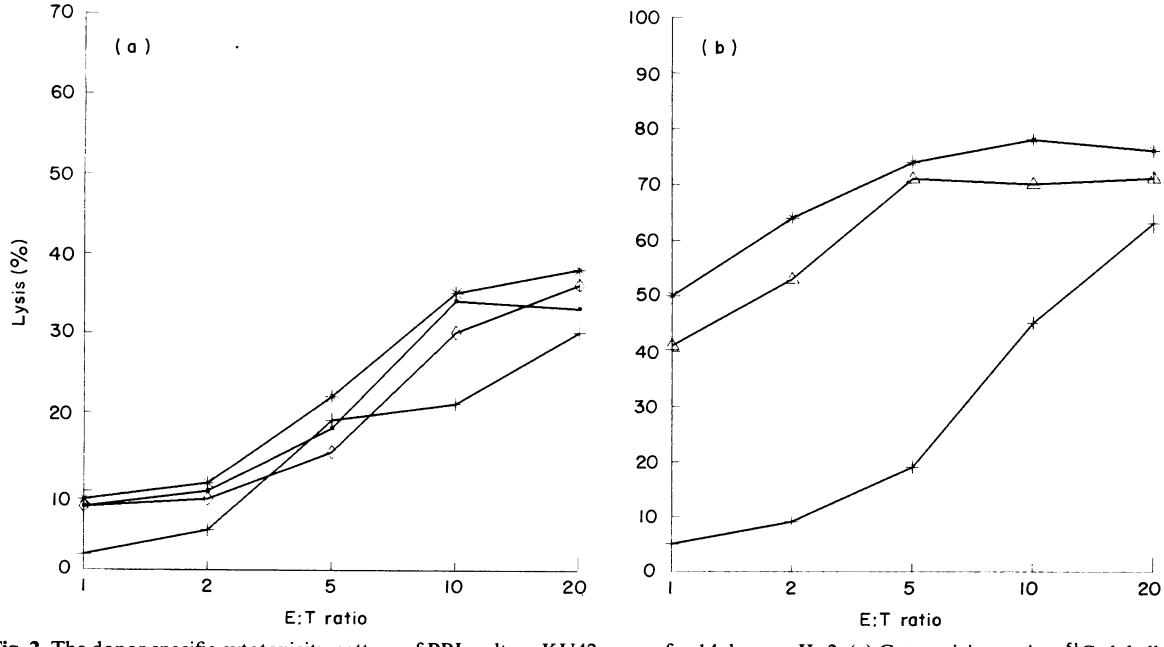

Fig. 2. The donor specific cytotoxicity pattern of PBL culture KU42 grown for 14 days on IL-2. (a) Cytotoxicity against ^{51}Cr-labelled donor B-LCL (■) could not be inhibited by a 10-fold excess unlabelled K562 (+), third-party T-BL-710 (*) or third-party B-LCL-VE (◇); (b) Cytotoxicity against labelled K562 (△) was inhibited by cold K562 (+) and not by cold third-party T-BL-710 (*).

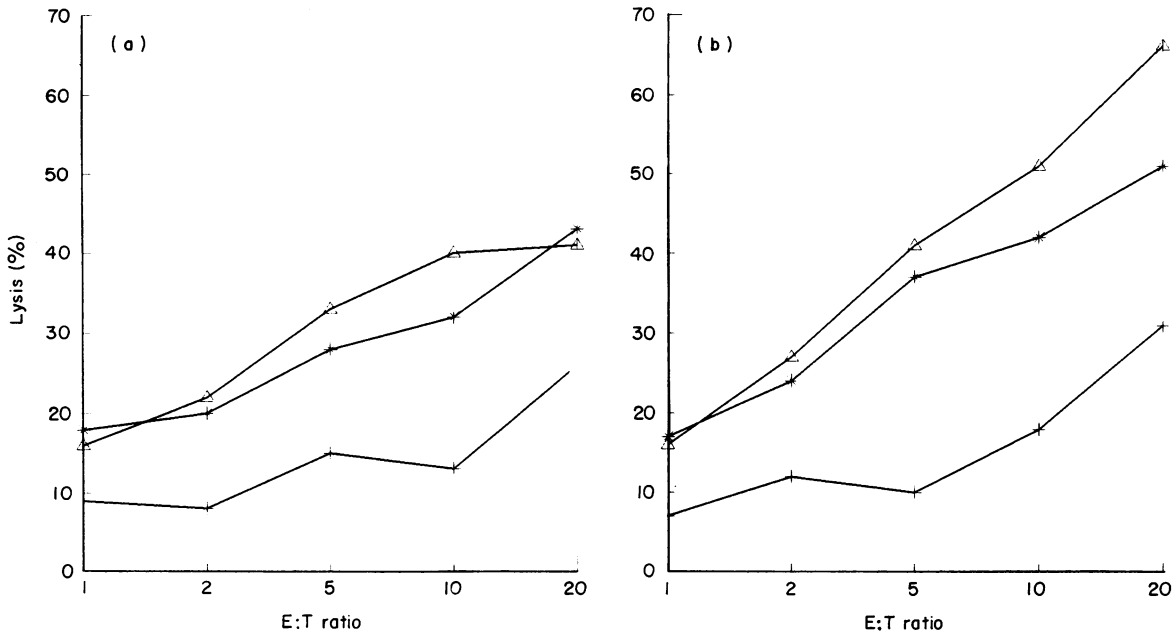

Fig. 3. LAK-like cytotoxic activity of culture WL132 against (a) labelled donor B-LCL (△) and (b) labelled third-party B-LCL (△) could be inhibited by a 10-fold excess unlabelled K562 (+) and not by a 10-fold excess cold third-party T-BL-78 (*).

the graft, nor in the episode just prior to rejection. In periods when no acute rejections were encountered and no cCTL were present in the graft, no donor-directed reactivity could be detected in the PBL. Similar results were reported by Suitters *et al.* [22] for humans and by Orosz *et al.* [23,24] for mice, using limiting-dilution assays (LDA). Both studies reported a higher frequency of donor-reactive CTL within the graft than in PBL or lymphnodes. Orosz *et al.* [23] used LDA methods that allowed for discrimination between cCTL and pCTL. Their results showed that the majority of CTL in the sponge allograft were cCTL, whereas in the spleen and regional lymph nodes the majority were pCTL. In our patient group mixed lymphocyte reactions (MLR) and MLR-induced CTL response against donor cells could be detected easily in PBL in the first 3 months post transplant (manuscript submitted). This indicates that although pCTL do circulate in PBL, cCTL are not detectable using the present approach. Reader *et al.* [25] suggested quantification of donor-reactive CTL in blood by means of LDA techniques as a useful tool for non-invasive rejection monitoring in cardiac transplants. An increase in the donor reactive CTL frequency in the blood was associated with signs of rejection in the graft. However, they could not discriminate between a histologic grade 1 (lymphocyte infiltrates) and grade 2 (infiltrates and myocytolysis) rejection. Most centres consider only grade 2 as clinically relevant and start rejection treatment when myocyte damage is seen in the EMB. Orosz *et al.* [23] suggested that enumeration of cCTL might be more informative. One major drawback for the use of LDA for peripheral rejection monitoring is the 10-day culture period. Another, more fundamental argument is that the peripheral blood may not be the preferential site for cCTL.

For the preferential accumulation of cCTL in the graft, three mechanisms might be involved: (i) pCTL migrate to the graft where they become activated by alloantigen and subsequently receive the lymphokine signal to become mature cCTL; (ii) after central sensitization, as postulated by Larsen *et al.* [26] and Austen & Larsen [27], immature CTL migrate from the lymphoid organs to the graft where they receive their final maturation step by lymphokines; and (iii) pCTL become fully activated to cCTL in the lymphoid organs, cCTL migrate at a very low frequency and accumulate in the graft where they expand under influence of IL-2. It is as yet unknown which mechanism takes place; however, the three possibilities are not mutually exclusive. The lymphokines may be produced in the graft by sensitized $CD4^+$ T cells.

We have reported previously [13] that CD4 cells indeed are dominant, especially in cultures propagated from EMB taken during acute rejection. From studies of Dallman *et al.* [28] it became apparent that a normal IL-2 pathway is required for allograft rejection. They showed that in the grafts of tolerant rats lymphocytic infiltrates were still present. However, those cells did not produce biologically active IL-2 and do not normally respond to IL-2.

If pCTL indeed undergo their final activation in the graft to become cCTL, it is obvious that monitoring of PBL can not be representative for intra-graft events. Our present data support this, and lead to the conclusion that at the moment EMB remain the only reliable source for the detection of cardiac graft rejection.

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